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# Transformed microorganisms with improved properties

## Field of the invention

The present invention relates to genetic engineering of production microorganisms used in biotechnology to improve their properties so that they produce useful products more efficiently. In particular the invention relates to increasing the yields of products such as ethanol or amino acids from carbon and nitrogen sources such as biomass comprising hexoses, pentoses or their polymers.

## Background of the invention

The efficiency of many biotechnological processes is limited by the need of production organisms to balance their metabolic redox reactions. In particular, for each of the pyridine nucleotide couples (NAD/NADH and NADP/NADPH) the total rate of oxidation must be equal to the total rate of reduction: otherwise, the couple will be completely converted into one form (e.g. all in the NAD form or all in the NADH form), and reactions requiring the other form will become infinitely slow, causing the whole metabolic network of reactions be distorted in an undesirable way (i.e., no longer provide the desired product).

For example, although the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are very efficient at converting hexoses into ethanol and have many advantages for this process (such as tolerance of high ethanol concentrations and other stresses) they are unable to ferment xylose to ethanol. Xylose is a major component of plants, and the inability to convert it to ethanol decreases the efficiency with which renewable biomass, such as agricultural wastes, can be utilized. However, these yeasts can utilize xylulose. Some yeasts (e.g. *Pichia stipitis*) can convert xylose to ethanol, although in poor yields, and contain the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH). These enzymes catalyse the sequential reduction of xylose to xylitol and oxidation of xylitol to xylulose. Transformed *S. cerevisiae* strains have been constructed containing heterologous XR and XDH, which so possess a pathway to convert xylose into the fermentable xylulose (Kötter and Ciriacy [1993]; Tantirungkij *et al.* [1994]; Walfridsson *et al.* [1995]). Although these strains could use xylose for growth and

xylitol formation, they did not produce much ethanol. All known XDH enzymes are specific for NAD, whereas all known XR enzymes are either specific for NADPH or have a preference for NADPH. It is believed (Bruinenberg *et al.* [1983]; Bruinenberg [1986]) that conversion of xylose to xylulose by this pathway therefore results in the cellular pool of NADPH being converted to NADP and that of NAD being converted to NADH, after which further metabolism of xylose is greatly hindered. The NADH can be reoxidised under aerobic conditions, but this demands critical control of oxygen levels to maintain fermentative metabolism and ethanol production. In contrast, bacteria that ferment xylose to ethanol efficiently contain xylose isomerase and convert xylose directly into xylulose without oxidation–reduction reactions. Attempts to create yeast that can efficiently convert xylose to ethanol have focused on finding or engineering XR or XDH enzymes with altered coenzyme specificity (Metzger and Hollenberg [1995]) or on expressing xylose isomerase gene in yeast. However, all reported attempts (see, e.g., Amore *et al.* [1989]; Ho *et al.* [1983]; Sarthy *et al.* [1987]; Walfridsson *et al.* [1996]) to construct good xylose–fermenting strains by expressing bacterial xylose isomerase genes in yeasts have failed.

As a second example, a major biotechnological process is the fermentation of hexose sugars to ethanol by yeast. The glycolytic pathway from glucose to ethanol is redox neutral, i.e. the amount of NAD reduced in the formation of a certain amount of pyruvate from glucose is exactly the same as the amount of NADH oxidised in the formation of ethanol from the same amount of pyruvate, and NADP(H) is not directly involved in the process. However, yeast growth is not a redox neutral process; the formation of 100 g dry yeast matter from glucose and ammonia is accompanied by the net production of 1.3 moles of NADH and 0.9 moles of NADP (Oura [1972]). This excess NADH is produced mainly by energy yielding catabolism, whereas the excess NADP is produced mainly by biosynthetic pathways (see Oura [1972]). Like other organisms, yeast has distinct pyridine nucleotide systems (NAD(H) and NADP(H)) that have evolved to facilitate these two aspects of metabolism. The excess NADH produced by fermenting yeast is reoxidised to NAD mainly by glycerol–3–phosphate dehydrogenase, resulting in the production of glycerol. In distillery fermentations this represents a wasteful diversion of 3–5 % of the carbon source (Oura [1977]). Attempts to decrease the proportion of glycerol to ethanol produced during fermentations have

met with little or no success. For example, Björkqvist *et al.* (1997) deleted each and both of the genes encoding glycerol–3–phosphate dehydrogenase. However, yeasts lacking this enzyme were not only unable to grow under anaerobic conditions, but they also stopped making ethanol.

A third example is the biotechnological production of amino acids. Amino acids have extensive applications in the food, animal feed, medical and chemical industries. Fermentation processes have been developed to produce most amino acids occuring in proteins. The metabolic routes to amino acids first convert a carbon source such as glucose into intermediates such as 3–phosphoglycerate, pyruvate, oxaloacetate or 2–oxoglutarate that are more oxidised than glucose. Their formation produces NADH. Most amino acids are more reduced than the intermediates, but the reactions leading to them from the intermediates almost invariably produce NADP. Apart from the histidine pathway, which is a net NADPH producer, and the pathways to glutamine, glutamate, tyrosine and phenylalanine, which neither consume nor produce NADPH, biosyntheses of all the other 15 amino acids from glucose produce between 1 and 8 moles of NADP per mole of amino acid and simultaneously produce NADH (Neidhardt *et al.* [1990]). Other reactions are then required to oxidise the NADH and reduce the NADP in order to achieve metabolic balance. This becomes a major factor with production organisms such as Corynebacteria modified and/or selected to produce huge amounts of amino acids on a commercial scale. To dispose of excess NADH, amino acid fermentations are operated under aerobic conditions, and oxygen is consumed in large amounts. To ensure maximum product formation, it is essential continuously to supply adequate amounts of oxygen, typically in the form of oxygen–enriched air (Hirose [1986]). Oxygen deficiencies, e.g., in high cell density fermentations or in cases where oxygen supplementation is uneconomical, typically result in lower product yields and productivities, as part of the carbon source is converted to compounds such as succinate, lactate or both to get rid of excess NADH.

Other examples include the enhanced biosynthesis of nucleotides, lipids and secondary metabolites by modified microorganisms selected or engineered to produce these compounds on the industrial scale. During these processes the microorganisms generally produce NADH and a central metabolic intermediate (such as pyruvate) that is more

oxidised than the carbon source and reduce this intermediate to the desired product using NADPH. Once again, the microorganisms need to oxidise the excess NADH and reduce the excess NADP, and the yields on carbon source are decreased by the additional metabolic transformations of the carbon source required to achieve redox balance.

In all these examples, excess NADH is reoxidised either by respiration, requiring efficient aeration, or by the formation of unwanted side products, such as glycerol. Aeration on large industrial scales is expensive, and difficult to control exactly. In some processes, such as the fermentation of xylose to ethanol, reduction of excess NADP causes at least as many problems. The most important biochemical reactions regenerating NADPH are the oxidative branch of the pentose phosphate pathway (PPP), i.e. the successive reactions of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and NADP-linked isocitrate dehydrogenase. Both reactions produce $CO_2$. In industrial scale operations, this represents both a direct loss of carbon source and an environmental pollution. Furthermore, $CO_2$ also acidifies culture media, necessitating the use of larger amounts of neutralising agents to control fermentation pH, and has a significant impact on cell physiology in general and amino acid production in particular. For example, $CO_2$ inhibits enzymes in methionine and purine biosynthesis and has been reported to inhibit product formation in several fermentation processes including production of isoleucine, inosine, fumarate, penicillin and other antibiotics and yeast biomass (Hirose [1986]).

A general method to alleviate these problems without using aeration, which is expensive and difficult to control at optimal levels, would be very beneficial. Potential benefits include increased yields on carbon source, decreased energy consumption and significant decreases in $CO_2$ production.

In the major routes of carbon and nitrogen metabolism it is a general rule that most catabolic pathways (often involved in energy production) use the NAD/NADH coenzyme couple in the oxidation-reduction steps, whereas anabolic, synthetic reactions more frequently use the NADP/NADPH couple. Although the redox potentials (E´o) of these two couples are both close to -0.32 (Kaplan [1960]), the ratios of the reduced and

oxidised forms of the two couples are maintained at very different levels in living cells. For example, in aerobic *S. cerevisiae*, NADH/NAD = 0.9 and NADPH/NADP = 3.2 (Lagunas *et al.* [1976])

Most pyridine nucleotide dehydrogenases have a marked, often nearly absolute, specificity for one or the other pyridine nucleotide. Some dehydrogenases with the same substrate occur as both NAD– and NADP–specific enzymes. Usually only one of the enzymes is present under certain conditions, or the enzymes are expressed in different cell compartments. Good examples are glutamate dehydrogenases which are subject to complicated control mechanisms usually resulting in only one of the enzymes being dominant under any growth condition (e.g. Courchesne and Magasanik [1988]; Coschigano *et al.* [1991]; Miller and Magasanik [1991]; ter Schure *et al.* [1995]; Dang *et al.* [1996]; Avendano *et al.* [1997]). Isocitrate dehydrogenase occurs as a mitochondrial NAD–linked enzyme and an NADP–linked enzyme that is found in the cytosol. Cells are therefore able to maintain NADH/NAD ratios much lower than the NADPH/NADP ratios, because reactions that transfer reducing equivalents between the two systems (and so would tend to equilibrate them) are restricted. Some bacterial and animal cells contain NAD(P) transhydrogenases (EC 1.6.1.1. and 1.6.1.2). Transhydrogenases are often membrane–bound enzymes with several subunits which are linked to energy production rather than to equilibration of the pyridine nucleotide systems. For the purposes of this patent application, the term "dehydrogenases" does not include the transhydrogenases EC 1.6.1.1 and 1.6.1.2. Many production organisms used in biotechnology, such as *S. cerevisiae* and Corynebacteria do not contain NAD(P) transhydrogenases, and so they appear to be unable to convert NADH *plus* NADP directly into NAD *plus* NADPH and *vice versa*.

The existence of two pyridine nucleotide systems and the absence of unregulated processes that would equilibrate them, suggests that the efficient growth and reproduction of presently evolved living organisms requires two distinct systems. The reason may be that a high NADPH/NADP ratio is required to drive biosynthetic reactions, whereas a lower NADH/NAD ratio is better suited for the generation of energy by pathways such as glycolysis and the tricarboxylic acid cycle (Metzler [1977]).

Boles *et al.* (1993) studied a mutant *S. cerevisiae* that lacked phosphoglucoisomerase, the enzyme that interconverts glucose–6–phosphate (Glc6P) and fructose–6–phosphate (Fru6P). This strain (a *pgi1*–deletion mutant) is unable to grow on any hexose or pentose, though it can grow on certain mixtures of fructose and glucose (e.g. 2 % fructose plus 0.1 % glucose). The authors found that transformation of the mutant with a genomic library prepared from the mutant itself resulted in certain transformants that were able to grow on glucose alone, although 3– to 4–times slower than wild type, and contained plasmids comprising the *GDH2* gene. This gene encodes an NAD–linked glutamate dehydrogenase. The authors argued that the simultaneous presence of substantial activities of both NADP– and NAD–linked glutamate dehydrogenases enabled the *pgi1*–deletion mutant to grow on glucose by metabolising it through the PPP and converting the resulting NADPH into NADH, which could then be re–oxidised by functional mitochondria. Thus, these mutants were proposed to convert NAD plus NADPH into NADH plus NADP, which is the opposite transformation to that required of industrial production microorganisms (see above). Furthermore, their ability to survive on glucose was strictly dependent on the presence of functional mitochondria and oxygen and they were unable to ferment sugars into ethanol (Boles *et al.* [1993]).

**Summary of the invention**

According to the present invention a microorganism such as a fungus or a bacterium is transformed with at least one recombinant DNA molecule encoding or otherwise causing the expression of at least one enzyme that facilitates an oxidation–reduction reaction between the two pyridine nucleotide coenzyme couples, NAD/NADH and NADP/NADPH. The enzyme or enzymes can be one or more members of a pair of pyridine nucleotide–linked dehydrogenases that have at least one common substrate but different pyridine nucleotide specificities. Biotechnological processes in which a net oxidation of one pyridine nucleotide coenzyme couple occurs together with a net reduction of the other are carried out more efficiently by the transformed microorganism according to the invention than by the corresponding non–transformed microorganism, and the aeration of such processes can be decreased and made more flexible. These processes include the fermentation of carbohydrate to ethanol by growing microorgan–

isms, the fermentation of xylose to useful products and the commercial production of amino acids, nucleotides, lipids and secondary metabolites by microorganisms.

Preferable microorganisms for the purposes of this invention are yeasts, filamentous fungi and bacteria. Preferable yeasts belong to the genus *Saccharomyces*, and are especially strains of the species *Saccharomyces cerevisiae*; the genus *Schizosaccharomyces*, and are especially strains of the species *Schizosaccharomyces pombe*; and the genus *Pichia*, and are especially strains of the species *Pichia stipitis*, as well as *Candida* spp. or *Pachysolen* spp. Useful filamentous fungi include e.g. *Trichoderma*, *Aspergillus*, *Neurospora*, *Fusarium*, *Paecilomyces* and *Penicillium*. From bacterial genera Corynebacteria are useful for the purposes of this invention, especially the strains *Corynebacterium glutamicum*, as well as Brevibacteria, such as *Brevibacterium flavum* and *B. lactofermentum*.

**Brief description of the drawings**

**Figure 1.** The genetic map of pAOS66 with the relevant genes, expression cassettes and restriction sites indicated.

**Figure 2.** The PCR product obtained in the amplification of the ORF YKL029C from the genome of *Saccharomyces cerevisiae*. Lane 1; λ DNA digested with *PstI* to obtain the following size markers (the most relevant ones are shown in bold); 14.0–11.5 kb, 5.0–4.5 kb, 2.8 kb, 2.5 kb, **2.14 kb**, **1.99 kb**, 1.7 kb, 1.16 kb, 1.09 kb, 0.81 kb and 0.52 kb+smaller. Lane 2; the PCR product obtained with the primers indicated in the text.

**Figure 3.** The PCR product obtained in the amplification of the gene fragment encoding the malic enzyme of *Aspergillus nidulans*. Lane 1; λ DNA digested with *PstI* to obtain the following size markers (the most relevant ones are shown in bold); 14.0–1.7 kb, 1.16 kb, 1.09 kb, 0.81 kb, 0.52 kb, 0.47 kb, 0.45 kb, 0.34 kb, **0.265–0.247 kb**, **0.216–0.200 kb**. Lane 2; the PCR product obtained with the primers indicated in the text.

**Figure 4.** Xylose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *GDH2* (H1803, solid symbols) and control strain (H1805, open symbols): comparison of growth and xylose utilization rates.

**Figure 5.** Xylose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *GDH2* (H1803, solid symbols) and control strain (H1805, open symbols): comparison of ethanol and xylitol production rates.

**Figure 6.** Xylose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *GDH2* (H1803, solid line) and control strain (H1805, dotted line): comparison of carbon dioxide evolution rates.

**Figure 7.** Specific enzymatic activities of NADP–glutamate dehydrogenase (NADP–GDH) and NAD–glutamate dehydrogenase (NAD–GDH) for strains H1805 (control) and H1803 at time points of 26 and 96 hours.

**Figure 8.** Glucose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *GDH2* (H1791, solid line) and control strain (H1793, dotted line): comparison of carbon dioxide evolution rates.

**Detailed description of the invention**

The central teaching of the present invention is that biotechnological processes can be enhanced by transforming production microorganisms with genes for enzymes that tend to equilibrate the two pyridine nucleotide systems that coexist in living cells. Surprisingly, although cells have evolved two distinct pyridine nucleotide systems, which are maintained at distinct redox potentials, and such equilibration reactions are apparently prohibited in naturally evolved cells, it was now found that these reactions promote the metabolic pathways desired for product formation by the engineered or selected microorganisms used in many biotechnological processes, and thereby benefit those processes.

In its first embodiment the present invention provides a microorganism which is transformed with at least one recombinant DNA molecule encoding or otherwise causing the expression of at least one of a pair of dehydrogenases with opposite coenzyme specificities for NAD/NADH and NADP/NADPH but at least one common substrate (S in equations (1) and (2)) in such a way that both members of the pair are simultaneously expressed in the same subcellular compartment, preferably the cytosol. The following reactions can then occur, which tend to equilibrate the NAD/NADH and NADP/NADPH coenzyme couples:

(1) $NADP + SH_2 \Longleftrightarrow S + NADPH$

(2) $S + NADH \Longleftrightarrow SH_2 + NAD$

Simultaneous operation of reactions (1) and (2) might be expected to proceed until the NAD/NADH and NADP/NADPH ratios are almost identical, because the redox potentials of the two couples are very similar. However, the inventors show here that when production microorganisms are transformed in this way, the efficiency with which raw material is converted into useful products, and in particular the yields of products on biomass are substantially increased.

Several pairs of dehydrogenases are known which share common substrates but have different pyridine nucleotide specificities. For example, there are both NAD- and NADP-linked forms of glutamate dehydrogenase (EC 1.4.1.2 and 1.4.1.4), isocitrate dehydrogenase (EC 1.1.1.41 and 1.1.1.42), aldehyde dehydrogenase (EC 1.2.1.3 and 1.2.1.4), alcohol dehydrogenase (EC 1.1.1.1 and 1.1.1.2), malate dehydrogenase (EC 1.1.1.37 and 1.1.1.82), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8 and 1.1.1.94), xylose-1 dehydrogenase (EC 1.1.1.175 and 1.1.1.179), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12 and 1.2.1.13), orotate reductase (EC 1.3.1.14 and 1.3.1.15) and ferredoxin reductase (EC1.18.1.2 and 1.18.1.3) but any appropriate pair of dehydrogenases may be used. Many dehydrogenases are known (see for example Enzyme Nomenclature 1992, Academic Press Inc.) and their properties can be found from the literature or determined by simple spectrophotometric assays (see, e.g. Bergmeyer [1974]). Besides naturally occuring enzymes with the desired pyridine nucleotide specificities, the invention also includes the use of genetically engineered enzymes with

altered pyridine nucleotide specificities. As an example of cofactor specificity changes, see e.g. Chen *et al.* (1994) and Chen *et al.* (1997).

The catalytic activities responsible for reactions (1) and (2) may occur in the same polymeric protein or even in a single polypeptide chain or be combined into such a polymeric protein or single polypeptide chain, for example by genetic engineering. The invention may also be realised by over-expressing a dehydrogenase that operates effectively with both pyridine nucleotide systems. Dehydrogenases that accept both pyridine nucleotides are known and include isozymes of glutamate dehydrogenase (EC 1.4.1.3), aldehyde dehydrogenase (EC 1.2.1.5) and alcohol dehydrogenase (EC 1.1.1.71). Little is known about how their activities are regulated *in vivo* so that they do not disturb the concentrations of pyridine nucleotides.

In its second embodiment the invention provides a microorganism which is transformed with at least one recombinant DNA molecule encoding or otherwise causing the expression of at least one enzyme that catalyses at least one step of a cyclic series of reactions in which NADP is reduced to NADPH and NADH is oxidised to NAD according to reactions (3) to (5):

(3) $NADP + SH_2 \Longleftrightarrow S + NADPH$

(4) $S + X \Longleftrightarrow Z + Y$

(5) $NADH + Z \Longleftrightarrow SH_2 + NAD$

In the direction written, reactions (3) to (5) convert NADP *plus* NADH to NADPH *plus* NAD, and in the opposite direction they carry out the opposite transformation. The enzymes catalysing reactions (3) and (4) are again a pair of dehydrogenases with a common substrate ($SH_2$) but opposite coenzyme specificities, as in the first embodiment of the invention, but now the reaction products (S and Z) are different. For the purposes of this invention, reaction (4) can be a series of steps instead of a single step, provided only that S is converted into Z without a net change in NAD or NADP. Thus, this series of reactions also tends to equilibrate the NAD/NADH and NADP/NADPH couples in the same way as reactions (1) and (2), except that this is now coupled to the transformation of $X \Longleftrightarrow Y$. Thus, at equilibrium it is not necessarily the case that the

NAD/NADH and NADP/NADPH ratios will be nearly equal: instead they will also depend on the equilibrium between X and Y. An example of this embodiment of the invention is provided by malic enzyme, pyruvate carboxylase and malate dehydrogenase, which catalyse the following reactions:

(6) *malic enzyme*: malate + NADP $\Longleftrightarrow$ pyruvate + $CO_2$ + NADPH

(7) *pyruvate carboxylase*: pyruvate + $CO_2$ + ATP $\Rightarrow$ oxaloacetate + ADP + Pi

(8) *malate dehydrogenase*: oxaloacetate + NADH $\Longleftrightarrow$ malate + NAD

Also in this embodiment of the invention, it is envisaged that a genetically engineered enzyme with changed coenzyme specificities can be used.

Thus in the first specific aspect of the invention, the host microorganism carries an XR enzyme which preferentially uses NADPH, and an XDH enzyme which preferentially uses NAD. The host microorganism can convert xylose into xylulose by these enzymes, but as described above, this process is inefficient and yields of ethanol on xylose are low or zero. An example of this aspect of the invention is provided in Example 8. An engineered strain of *S. cerevisiae* carrying genes for XR and XDH, and xylulose kinase (XK), is transformed with a multicopy plasmid carrying the gene *GDH2* encoding the NAD-dependent glutamate dehydrogenase from *S. cerevisiae*, and a marker gene. Transformants are selected by means of the marker gene. The transformants ferment xylose to ethanol more efficiently than the non-transformed host yeast, in particular with a higher yield of ethanol on xylose, or less $CO_2$ production or both. Depending on the chosen process conditions, the improved efficiency can also be realised in other ways, such as an increased volume productivity or enhanced specific rate. This may be explained by the following sequence of reactions:

(9) xylose + NADPH $\Rightarrow$ xylitol + NADP

(10) xylitol + NAD $\Rightarrow$ xylulose + NADH

(11) NADH + 2-oxoglutarate + $NH_3$ $\Rightarrow$ glutamate + NAD

(12) NADP + glutamate $\Rightarrow$ 2-oxoglutarate + $NH_3$ + NADPH

SUM: xylose $\Rightarrow$ xylulose

Thus, the redox imbalance is avoided, and a smooth conversion of xylose to xylulose can take place. The flux through decarboxylation reactions, such as G6PDH and isocitrate dehydrogenase to regenerate NADPH is decreased, with decreased $CO_2$ production, and the fermentation occurs efficiently, and without aeration.

In this example, the host microorganism had already been transformed with genes encoding XR, XDH and XK. It is not a requirement of the invention that the host organism is itself a transformant. It is remarkable that the invention causes a substantial increase in ethanol yields with the host microorganism of Example 8, because the XR in this host is the enzyme from *P. stipitis*, which is able to work with NAD(H) although it has a preference for NADP(H) (Verduyn *et al.* [1985]). Thus, this aspect of the invention is realised even with an XR that can use NADH. A more substantial effect can occur when the host organism contains an XR with higher specificity for NADP(H), such as when the *S. cerevisiae* open reading frame XHR 104w encoding XR activity is expressed at a higher level than naturally. Furthermore, it has been claimed that transformation of yeasts with an XK encoding gene improves the efficiency with which they ferment xylose to ethanol (Ho and Tsao, WO 95/13362). It is notable that the present invention causes an improved fermentation of xylose to ethanol even when the host organism contains elevated levels of XK. However, the invention provides improved fermentation of xylose to ethanol also in host organisms that have not been transformed with a gene encoding XK.

It will be understood by a person skilled in the art that the same beneficial effect can be obtained by using other pairs of dehydrogenases according to the first embodiment of the invention described above, or by using appropriate enzymes according to the second embodiment of the invention.

Various enzymes, including isomerases and epimerases, are known that interconvert the different pentose sugars and different pentose phosphates. It will be understood by a person skilled in the art that the present invention provides a general method to improve the efficiency of ethanol production, not only from xylose but also from other pentoses.

For example, instead of transforming *S. cerevisiae* with *GDH2* so that both NAD– and NADP–linked glutamate dehydrogenases are adequately expressed in the cytosol (non–transformed *S. cerevisiae* already expresses the NADP–linked glutamate dehydrogenase), the same effect can be achieved by transforming the yeast with one or both members of another pair of dehydrogenases that share the same substrates but use different pyridine nucleotides, provided that both of the enzymes are reversible, or at least that they catalyse the reactions in the directions shown in equations (11) and (12). For example, most NAD–linked isocitrate dehydrogenases are allosteric enzymes that cannot catalyse the reductive carboxylation of 2–oxoglutarate (corresponding to reaction (11)), but only the oxidative decarboxylation of isocitrate, and would therefore be unsuitable for this aspect of the present invention. However, several other pairs of dehydrogenases can be used, including alcohol and aldehyde dehydrogenases. Appropriate information can be obtained from the literature or readily determined by testing with simple spectrophotometric assays. The activities of dehydrogenases can be easily measured in both directions, provided that they are reversible, by following the appearance or disappearance of NAD(P)H in the presence of the appropriate substrates to determine whether candidate enzymes catalyse the reactions required to relieve the coenzyme imbalance.

According to the second embodiment of the present invention, the same beneficial effect could be obtained by transforming *S. cerevisiae* with a recombinant DNA molecule encoding an NADP–linked malic enzyme directed to be expressed in the cytosol. *S. cerevisiae* already contains pyruvate carboxylase and malate dehydrogenase in the cytosol. The yeast can now catalyse the reactions (6) to (8) as shown above, resulting in the conversion of NADP *plus* NADH into NADPH *plus* NAD.

In another specific aspect of the invention, the host microorganism ferments hexose sugars to ethanol. Because the microorganism grows during the fermentation it produces excesses of both NADH and NADP (Oura, [1972]). With the non–transformed microorganism, ethanol production is accompanied by glycerol production, which is required to reoxidise the excess NADH, and by the production of more than one mole of $CO_2$ per mole of ethanol, which is required to reduce the excess NADP. These reactions decrease the yield of ethanol on fermentable carbohydrate. With the transformed microorganism,

the yield of ethanol on fermentable carbohydrate is increased compared to that of the untransformed microorganism. An example of this aspect of the invention is provided in Example 9.

The yeast *S. cerevisiae* is transformed with a multicopy plasmid comprising the gene *GDH2* encoding the NAD-dependent glutamate dehydrogenase from *S. cerevisiae* and a marker gene. The transformants are selected by means of the marker gene. The transformants ferment glucose and other hexoses to ethanol with improved efficiency, in particular an improved yield of ethanol on fermentable carbohydrates and with decreased production of some unwanted side products, including $CO_2$. Depending on the chosen process conditions, the improved efficiency can also be realised in other ways, such as an increased volume productivity or increased specific rate. This may be explained by the following sequence of reactions that converts substantial parts of the excess NADH and NADP into NAD and NADPH without unwanted consumption of fermentable sugars:

$$\text{NADH} + \text{2-oxoglutarate} + NH_3 \Rightarrow \text{glutamate} + \text{NAD}$$

$$\text{NADP} + \text{glutamate} \Rightarrow \text{2-oxoglutarate} + NH_3 + \text{NADPH}$$

It will be understood by a person skilled in the art that the same beneficial effect on hexose fermentation to ethanol can be obtained by using other pairs of dehydrogenases according to the first embodiment of the invention disclosed above or by using appropriate enzymes according to the second embodiment of the invention, as has been disclosed above in connection with improved fermentation of xylose to ethanol.

It is a significant part of the invention that the same transformation that increases the efficiency of xylose fermentation to ethanol (Example 8) also increases the efficiency of hexose fermentation to ethanol (Example 9). Thus, the invention simultaneously provides improved utilization of both glucose and xylose, which are major sugars derived from many renewable biomasses, such as agricultural and forest materials and urban waste.

In a third specific aspect of the invention a microorganism that overproduces an amino acid such as alanine, valine, leucine, isoleucine, serine, threonine, lysine, arginine, tryptophan, cysteine, methione or proline is transformed with at least one recombinant DNA molecule encoding at least one of a pair of dehydrogenases with opposite pyridine nucleotide specificities (i.e., according to the first embodiment of the invention), or with at least one recombinant DNA molecule encoding at least one enzyme that catalyses at least one step of a cyclic series of reactions in which NADP is reduced to NADPH and NADH is oxidised to NAD according to reactions (3) to (5) (i.e. according to the second embodiment of the invention). The transformed microorganism can produce the desired amino acid with improved efficiency, in particular with increased yield on carbon source, with increased productivity, with a decreased requirement for aeration, with decreased production of carbon dioxide or with several of these benefits.

For example, lysine is presently produced by microbial fermentation processes that are mainly based on various Corynebacteria, such as *Corynebacterium glutamicum* and *Brevibacterium flavum*. Genetic techniques for these bacteria are well developed (see, e.g., Follettie *et al.* [1991]; Follettie & Sinskey [1986]; Jetten *et al.* [1994]; Jetten & Sinskey [1995]) and DNA vectors are available for the transformation of these production organisms efficiently by either multicopy plasmids or chromosomal integration. For example, available vectors include pAJ655, pAJ1844 and pCG11 for use with *C. glutamicum, Brevibacterium* spp., and *Escherichia coli* or the pAJ440 plasmid vector for use in *Bacillus subtilis, Brevibacterium* spp., and *C. glutamicum*, or the pMS2 plasmid vector for use in *Rhodococcus* spp., *Corynebacterium* spp., and *E. coli.* The effects of transformation of lysine-producing strains according to the present invention can be realised in strains such as ATCC 31269, ATCC 21253, ATCC 21800, ATCC 21801 or ATCC 21086 or in other strains that over-produce lysine or other amino acids and are used industrially.

Other aspects of the invention include the transformation of production microorganisms that have been developed to overproduce nucleotides, lipids or secondary metabolites of various types on an industrial scale. After transformation according to the above described embodiments of this invention, these microorganisms provide increased yields of the desired commercial products.

These examples disclose how the efficiency of biotechnological processes can be improved by transforming production microorganisms with at least one recombinant DNA molecule encoding enzymes that facilitate oxidation–reduction reactions between the NAD(H) and NADP(H) coenzyme systems. The invention can be realised by transforming the host with a single gene (e.g. *GDH2* in Example 3) and using enzymes that are naturally expressed in the host under the specific production conditions to complete the reaction schemes (i.e. reactions (1)+(2) or (3)+(4)+(5) above). However, it should be understood that the invention can also be realised by transforming the host with more than one DNA–molecule so that both reactions (1) and (2) or two or more of the reactions (3), (4) and (5) are performed by enzymes expressed from transformed genes.

Besides *GDH2* and a gene encoding malic enzyme, genes encoding other enzymes can be advantageous. Suitable enzymes (examples are given above) are known and their genes have been cloned and can be found in data banks and obtained by PCR methods well known in the art. Examples of potentially useful genes are those encoding the NADP(H) utilizing counterparts of the reaction with Swissprot accession numbers P54115, P00369, P31026, P00370, P50216, P08200, P14941, P75214, P27800, Q58820, P15719, P46919, P28861, L05667, U26463, YPL061w, P18819, M88600, X75327 and P55804, which can be simply cloned, e.g., by PCR with sequence specific primers. For example, the *S. cerevisiae* malic enzyme can be cloned as demonstrated in Example 6, and coupled to a vector and transformed into an appropriate microbial host using methods known in the art.

Other suitable enzyme activites can be found by carrying out appropriate enzyme assays (see, e.g., Bergmeyer [1974], but other suitable assay systems can be readily designed by a person skilled in the art) on extracts prepared from suitable organisms, including bacteria, fungi and higher plants and animals. The responsible protein can then be purified by standard methods, and antibodies prepared against it or amino acid sequence data obtained from it. The gene encoding the protein can then be cloned by standard methods such as using antibodies to screen expression libraries or oligonucleotides designed from the amino acid sequences to act as primers in PCR cloning or hybridization probes to screen gene banks.

Suitable new enzyme activities and their genes can also be found by exploiting data bank information in other ways. For example, alignment of several sequences encoding malic enzymes reveals a so called "malic enzyme signature", which allows the preparation of oligonucleotide mixtures that can be used, for instance, in PCR cloning of genes encoding malic enzymes in other organisms, as is described in Example 7 for the malic enzyme of *Aspergillus nidulans*.

According to the present invention, the host organism is transformed in such a way that the reactions (1) and (2) (in the first embodiment of the invention) or the reactions (3) to (5) (in the second embodiment) occur simultaneously in the same subcellular compartment, preferably the cytosol. The invention thus teaches that the transforming gene is modified if necessary to cause expression in the appropriate compartment and under the physiological conditions prevailing during the desired production process. So called "signal" or "targetting" sequences are known that usually encode relatively short N-terminal or C-terminal amino acid sequences that direct proteins to specific compartments such as mitochondria, peroxisomes or periplasmic space (McAlister-Henn *et al.* [1995]). These sequences can be readily removed or added to genes by standard techniques of genetic engineering to cause the desired enzymes to be expressed in the desired compartment. In addition, enzymes subject to catabolite inactivation can be modified to slow or prevent this regulatory circuit (Minard and McAlister-Henn [1992]).

Thus, the present invention can be practised by transforming a microorganism with a recombinant DNA molecule so that the natural promoter of a host gene encoding a suitable dehydrogenase is replaced by another promoter that can cause stronger expression or expression under different physiological conditions than the said natural promoter. In this embodiment of the invention, it is not necessary that the transforming DNA molecule contains a nucleotide sequence encoding a complete functional dehydrogenase. For example, the beneficial effect can be obtained by transforming the host *S. cerevisiae* with a DNA molecule that only replaces through recombination *in vivo* the natural promoter of the host's *GDH2* with a promoter such as PGK or ADH, so that the host's NAD-dependent glutamate dehydrogenase is constitutively expressed.

When the host is transformed with a gene from another organism, it is desirable to use a promoter derived from the host.

For many production fungi and bacteria suitable promoters are known. Examples include the *S. cerevisiae* PGK, ACT, ENO1, GAPDH, MET, such as MET25 and ADH promoters, and modified versions thereof (e.g. Ruohonen *et al.* [1995]; Beier and Young [1982]). It is envisaged in the invention that, in the case of *S. cerevisiae* for example, use of these promoters can be advantageous even when the transformed gene or genes are obtained from yeast. They can be advantageous especially when the genes are to be integrated into the host's genome, because these promoters are known to cause adequate expression under a range of physiological conditions. For example, the so called middle length ADH1 promoter causes efficient expression in *S. cerevisiae* under both fermentative and gluconeogenic growth conditions. However, adequate expression of the transformed gene or genes is also possible to be obtained with the genes' natural promoters, for example, by transformation with a multicopy plasmid, as disclosed in Examples 3 to 5. This type of effects can also be obtained by modifications of the promoter in question or by modifications of the transacting regulatory mechanisms (negative or positive) involved in the expression of the particular gene.

When foreign genes are transformed into an organism, it is desirable to transform with a DNA sequence without introns, obtained for example from cDNA or by artificial synthesis.

Any method available for introducing or transforming genes into the host is suitable for this invention and various types of vectors can be used, including autonomously replicating plasmid vectors or artificial chromosomes. Methods described in the art to integrate single or multiple copies of transforming genes into chromosomes in functional, expressible forms are also suitable for this invention. Examples of such methods for yeast, filamentous fungi and Corynebacteria, and other microorganisms have been described. An appropriate marker gene can be included in the transforming vector so that transformants can be easily selected. A wide range of marker genes is known. Transformants can also be selected by expression of a desired phenotype, such as enhanced ability to grow on xylose under anaerobic conditions (see Example 8).

It is envisaged in the invention that it can be advantageous in some cases to cause expression of the transformed genes only under specific culture conditions. For example, it can be useful first to grow the organism to a certain cell density, and then cause expression of the transforming gene. Promoters are known that can be induced by changes in temperature or pH, by particular carbon or nitrogen sources or by the presence or absence in the medium of certain organic or inorganic substances, such as phosphate or copper. Examples of yeast promoters that have been used for such inducible expression include GAL1, GAL10, CUP1 and PHO5.

The present invention is further illustrated by the following Examples which describe construction of the production strains of the invention, as well as their use in the above indicated specific aspects of the invention. If not otherwise indicated, all biotechnological procedures are carried out using methods conventional in the art.

**Example 1. Construction of the integrant strain with the *XYL1* and *XYL2* genes of *Pichia stipitis* encoding xylose reductase and xylitol dehydrogenase**

The pMA91 (Mellor *et al.* 1983) based yeast expression vector pAOS66 (Figure 1) containing the *XYL1* under the *PGK1* promoter and the *XYL2* under the modified *ADH1* promoter (Ruohonen *et al.* [1995]) was digested with *HindIII* to isolate the 2.8 kb expression cassette carrying the *XYL1* gene between the promoter and terminator of *PGK1* and with *BamHI* to isolate the 2.2 kb expression cassette carrying the *XYL2* gene between the modified *ADH1* promoter and *ADH1* terminator. Plasmid B955 (Toikkanen *et al.* 1998) was used to construct the integration cassette. B955 is the Bluescript SK bacterial cloning vector (Stratagene) carrying two fragments of the *URA3* gene (encoding orotidine–5'–P decarboxylase, Rose *et al.* 1984); base pairs 71–450 and 781–1135 from the encoding region of the gene at *SacI–XbaI* sites and *XhoI–Asp718* sites, respectively, of the polylinker region. The remaining polylinker sites *HindIII* and *BamHI* in the cloning vector were used for introducing the *XYL1* and *XYL2* expression cassettes between the two *URA3* fragments by sticky–end ligations. The resulting construction (5' *URA3* 71–450 bp – *XYL2* expression cassette 3'–5' – *XYL1* expression cassette 5'–3' – *URA3* 781–1135 3') was released from Bluescript SK by *SacI–NsiI* digestion and isolated from an agarose gel. One $\mu$g of the fragment was used to

transform the yeast strain CEN.PK2 (VW-1B) (*MATα leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8$^c$ SUC2*) (Boles *et al.* [1996]) by the LiAc transformation procedure (Hill *et al.* [1991], Gietz *et al.* [1992]). The strain CEN.PK2 (VW-1B) is called "strain H1346" by us, and it has a VTT strain collection number VTT C-98304.

The integration strategy is based on the toxicity of 5-FOA (5-fluoro-orotic acid) to the yeast cells (Boeke *et al.* [1984]). Wild type cells convert 5-FOA to 5-FUMP (5-fluoro-uridine monophosphate), a potent inhibitor of thymidylate synthetase. Thus only *ura3* (and *ura5*) mutants can grow in the presence of 5-FOA, as long as uracil is provided for the mutant strain. Integration of the above described fragment into the *URA3* locus disrupts the wild type gene and the strain becomes uracil auxotrophic, allowing it to grow on 5-FOA plates.

The correct, functional integration was verified by Southern blotting, by measuring the XR and XDH activities in cell extracts and by showing that the integrant strain only grew on xylose in shake flask cultivations, as compared to the non-transformed CEN.PK2 (VW-1B) strain. The integrant strain was named as H1469.

**Example 2. Cloning of *Saccharomyces cerevisiae* xylulokinase gene (SGD no. YGR 194C)**

The xylulokinase gene (*XK*) was amplified from total DNA of wild type yeast strain by PCR, using forward primer 5' CCA GTG ATA TCG AGG ATG AGA TTA GTA C 3' and reverse primer 5' CCA GTG ATA TCT GTA CTT GTC AGG GCA T 3'. Both primers contain an *EcoRV* restriction site at the 5' end. PCR reaction conditions were: 94°C 3' hot start; 94°C 1', 55°C 1', 72°C 2', 30 cycles, 72°C 10' final extension. The PCR product was digested with *EcoRV* and purified from an agarose gel. The *XK* fragment was ligated into the vector B609 (Bluescribe M13; Stratagene, with the modified *ADH1* promoter and *ADH1* terminator) which had been treated with Klenow enzyme to make blunt ends. Orientation of the fragment was checked with *BglII* and *EcoRI* enzymes. A clone with the right orientation was digested with *BamHI* and the fragment was purified from an agarose gel. The *BamHI* fragment was cloned to *BamHI* site of YEplac195 yeast expression vector (Gietz and Sugino [1988]).

**Example 3. Cotransformation of the integrant strain H1469 with the genes encoding xylulokinase (XK) and NAD-dependent glutamate dehydrogenase (NAD-GDH) on two separate multicopy expression vectors**

Two yeast expression vectors, the above described YEplac195 carrying the gene encoding the xylulokinase and YEplac181 carrying the gene *GDH2* encoding the NAD-dependent glutamate dehydrogenase (Boles *et al.* [1993]) were cotransformed into H1469 integrant strain. YEplac195 vector was selected for by omitting uracil and YEplac181 by omitting leucine from the growth medium. Plasmid rescue from the yeast transformants verified the integrity of the two expression plasmids. The strain carrying both XK and NAD-GDH encoding genes was named as H1803 (VTT C-98302). A control strain carrying the gene encoding XK and YEplac181 control plasmid without *GDH2* was named as H1805 (VTT C-98303).

**Example 4. Transformation of the integrant strain H1469 with the gene encoding NAD-dependent glutamate dehydrogenase on a multicopy expression vector**

The above described plasmid YEplac181 carrying the gene *GDH2* encoding the NAD-dependent glutamate dehydrogenase was transformed into the integrant strain H1469. Selection of the transformants was as mentioned above. Plasmid rescue from the yeast transformants verified the integrity of the expression plasmid. The strain carrying the gene encoding NAD-GDH was named as H1795 (VTT C-98300). A control strain carrying the YEplac181 control plasmid was named as H1797 (VTT C-98301).

**Example 5. Transformation of the yeast strain H1346 (CEN.PK2 (VW-1B)) with the gene encoding NAD-dependent glutamate dehydrogenase on a multicopy expression vector**

The above described plasmid YEplac181 carrying the gene *GDH2* encoding the NAD-dependent glutamate dehydrogenase was transformed into the CEN.PK2 (VW-1B) (=H1346) strain. Selection of the transformants was as mentioned above. Plasmid rescue from the yeast transformants verified the integrity of the expression plasmid. The strain carrying the gene encoding NAD-GDH was named as H1791 (VTT C-98298). A

control strain carrying the YEplac181 control plasmid was named as H1793 (VTT C–98299).

**Example 6. Cloning of the open reading frame YKL029C encoding the malic enzyme homologue from *Saccharomyces cerevisiae***

The malic enzyme has been characterized from *S. cerevisiae* (Fuck *et al.* 1973). Analysis of the yeast genome revealed one open reading frame (ORF YKL029C) with homology to the gene encoding malic enzyme from *Schizosaccharomyces pombe* (Viljoen *et al.* 1994). The *S. cerevisiae* ORF YKL029C was amplified from the yeast chromosomal DNA by PCR using forward primer 5' CAT GCT AAG CTT CTA GAA TGC TTA GAA CCA GAC TA 3' and reverse primer 5' GAT GCT AAG CTT CTA GAT GGT TAT GCT TCG TCT AC 3'. Both primers contain *HindIII* and *BglII* restriction sites at the 5' end. PCR reaction conditions were: 94°C 3' hot start; 94°C 1', 40°C 1', 72°C 2', 30 cycles, 72°C 10' final extension. The DNA fragment obtained was of expected size (2.0 kb, Figure 2). The PCR fragment was digested with the appropriate restriction enzyme (*BglII*) to allow its cloning between the promoter and the terminator of *PGK1* in the yeast expression vector pMA91, and transformed into yeast strains CEN.PK2 (VW–1B) (H1346) and H1805 (but not containing YEplac181), resulting in strains H1840 and H1842, respectively.

**Example 7. Cloning of the gene encoding the malic enzyme from the filamentous fungus *Aspergillus nidulans***

All sofar cloned genes from different organisms encoding the malic enzymes contain a DNA sequence coding for the "malic enzyme signature". It is a highly conserved, unique amino acid sequence (FNDDIQGTGAVVMASLI) of this particular protein (Prosite: PDOC00294). The signature allows specific degenerated primers to be planned for cloning of any particular gene encoding a malic enzyme.

Degenerated primers were planned using the malic enzyme signature (region D) for the 3' end primer and a second homologous region of the protein, the region C for the 5' end primer (Viljoen *et al.* 1994). The forward primer was 5' GA(T/C) GTI GGI ACI

AA(T/C) AA 3' and the reverse primer was 5' GTI CC(T/C) TG(A/G/T) AT(A/G) TC(A/G) TC(A/G) TT(A/G) AA 3'. PCR reaction conditions were 94°C 3' hot start; 94°C 1', 37°C 1', 72°C 2', 7 cycles, 94°C 1', 40°C 1', 72°C 2', 25 cycles, 72°C 10' final extension Chromosomal DNA of *Aspergillus nidulans* was used as the template in the PCR reaction. A fragment of expected size was obtained (0.24 kb, Figure 3).

**Example 8. Ethanol Production from Xylose**

**Part 1. Shake flask cultivations**

The strains H1803 and H1805 (see Example 3) were cultivated in the growth medium which was modified SC–URA–LEU (synthetic complete media, uracil and leucine omitted, Sherman *et al.* [1983]) and Yeast Nitrogen Base without Amino Acids (Difco) and the carbon sources D–glucose (20 g/l) or D–xylose (20 g/l). Cells were pre–grown in shake flasks in a medium containing glucose as a carbon source. Cells were collected by centrifugation and resuspended in a volume of 100 ml media containing xylose as a carbon source. Cells were kept at 30 °C in 100 ml Erlenmeyer's gently stirring with a magnetic rod. Anaerobiosis was achieved by using an airlock.

After two days the strain with elevated levels of NAD–GDH had produced 2.35 g ethanol / mg dry weight, and the control strain produced 1.47 g ethanol / mg dry weight. The amount of ethanol was measured enzymatically with the aid of an auto–mated analyser (Cobas – Mira).

Glutamate dehydrogenase activity was measured in a crude yeast cell extract, which was obtained by vortexing 500 mg of fresh cells in 500 µl of 100mM Na–phosphate, pH 7.0 and 1 g glass beads (diameter 0.4 mm) for 15 minutes. The mixture was then centri–fuged in a table top centrifuge and the supernatant assayed. 200 µl of a buffer contain–ing 200 µM NAD(P)H and 100 mM Na–phosphate, pH 7.4 were mixed with 10 µl of the 20 fold diluted crude yeast extract. To start the reaction α–ketoglutarate (final concentration 10 mM) and ammonium chloride (final concentration 20 mM) were added. The rate of decreasing absorbance at 340 nm was measured and the activity was calculated from this. It is related to the protein content of the yeast extract as measured by a BIORAD protein assay. The NADP–GDH activity was measured using NADPH as

a substrate and the NAD–GDH activity was measured with NADH as a substrate. The NAD–GDH activity was estimated 4 – 5 nkat/mg in the overexpression strain, and 0.04 nkat/mg in the control without *GDH2* overexpressed. The NADP–GDH activity was about 2 nkat/mg. All assays were performed in a Cobas – Mira automated analyser.

**Part 2. Fermentor cultivations**

Anaerobic xylose fermentation to ethanol was conducted in 1.8 liter Chemap CMF Fermentor (Switzerland) by genetically engineered strains of *Saccharomyces cerevisiae*, designated as **H1803** and **H1805**. Both strains are derived from *S. cerevisiae* CEN.PK2 (VW–1B) (Boles *et al.* [1996]), and express xylose reductase (XR) and xylitol dehydrogenase (XDH) by chromosomal integration of the corresponding genes from *Pichia stipitis*. In addition, both strains overexpress the native xylulokinase (XK) from a multicopy plasmid (YEplac195+*XK*). Strain H1803 contains an additional plasmid that expresses the NAD–glutamate dehydrogenase (NAD–GDH) from *S. cerevisiae* (YEplac181+*GDH2*), whereas strain H1805 contains only the cloning vector (YEplac181) and serves as a control strain. Omitting uracil (URA) from the growth media can minimize plasmid segregation for the YEplac195 vector, and leucine (LEU) for YEplac181.

The seed cultures of strains H1803 and H1805 were routinely maintained on plates that were renewed every 2–3 weeks. The pre–inoculum was prepared by transferring a single colony into a 250 ml Erlenmeyer flask that contained 50 ml of modified synthetic complete medium without uracil and leucine (SC–URA–LEU) + 20 g/l of glucose (Sherman *et al.* [1983]). For each strain three identical flasks were prepared. The cells were grown overnight on a rotary shaker at 150 rpm and 30 °C, and the content of each flask was then transferred completely into a 3 l baffled flask that contained 500 ml of SC–URA–LEU plus 50 g/l of glucose and grown aerobically at 150 rpm and 30 °C until glucose was exhausted ($OD_{600}$: 20–25).

Cells from the above cultivation (six flasks) were harvested by a 10 minute centrifugation at 4,500 rpm and 4°C, washed by a 0.1 M $PO_4^{2-}$ buffer (pH=5.5) and resuspended in the same buffer each to a final volume of 30 ml and subsequently transferred to the fermentor. The fermentation medium contained SC–URA–LEU +

10% xylose. The fermentor temperature was maintained at 30 °C, the pH was controlled at 5.5 by addition of 2 M NaOH, and the agitation was constant at 300 rpm. The cultivation was carried out under anaerobic conditions by flashing the headspace of the fermentor with nitrogen at a constant flow rate of 0.2 vvm. The offgas was connected via a multi-port valve to a Balzers quadrupole mass spectrometer (Sweden) for online analysis. Liquid samples were withdrawn from the fermentor at time intervals to measure growth, substrate consumption, and the formation of extracellular products. For selected samples the activities of the NADP- and NAD-glutamate dehydrogenases were also measured by standard enzymatic techniques. Growth was monitored by measuring both the absorbance at 600 nm, as well as the dry cell weight (DCW) by filtration and subsequent drying to constant weight. Xylose, ethanol, xylitol, glycerol and acetate present in the fermentor broth were separated on an HPX-87H column (55 °C), with 5 mM $H_2SO_4$ as eluent (0.6 ml/min), and quantified by refractive index (RI) detection. The amounts of ethanol, glycerol, xylitol and acetate were independently verified by appropriate enzymatic assays with the aid of an automated analyser (Cobas - Mira).

The growth and xylose consumption of strains H1803 and H1805 for the first 30 hours of cultivation are summarized in Figure 4. Both strains can ferment xylose effectively at comparable rates, however, the NAD-GDH overexpressing strain (H1803) accumulates about 6% less biomass (7.13 vs. 6.72 g/l). The most remarkable difference between the two strains is ethanol production. As illustrated in Figure 5, by the end of the 30-hour time period the *GDH2* strain accumulates about 1.02g ethanol per g DCW compared with 0.73 for the control strain. This represents an enhancement of specific ethanol production of approximately 40% for the *GDH2* strain (0.58 vs. 0.83 mmol/g-cell h). The volumetric productivity is also higher for the *GDH2* strain by about 30% (3.94 vs. 5.20 mmol/l h). The corresponding yields of ethanol on xylose are 0.21 and 0.29 (mol/mol) for the control and *GDH2* strains, respectively. Unexpectedly, xylitol production was also elevated for the *GDH2* strain by about 25% as shown again in Figure 5.

Yet another extraordinary divergence between the two recombinant strains is depicted in Figure 6. This carbon dioxide data comes from the mass spectrometer measurements of

the effluent gas. As shown in Figure 6, overexpression of *GDH2* significantly attenuates carbon dioxide evolution. Integrated values for $CO_2$ production from 0 to 30 hours are 100.4 and 80.7 mmol/l for the control and *GDH2* strains, respectively, i.e. the *GDH2* strain wastes about 20% less of the carbon source to carbon dioxide production.

Enzymatic assays for NADP–GDH and NAD–GDH were performed on chosen samples and results from two such sets are shown in Figure 7. The assay mixture contained 100 mM sodium phosphate buffer at pH=7.0, 200 $\mu$M of either NADPH (NADP–GDH assay) or NADH (NAD–GDH assay), and cell lysate at a final concentration of about 0.5 mg/ml. The reaction was started by addition of 20 mM α–ketoglutarate plus 40 mM $NH_4Cl$, and the NAD(P)H consumption was monitored spectrophotometrically at 340 nm. Both strains have notable NADP–GDH activities as expected, although H1803 appears to have a surprisingly higher specific activity for this enzyme (about 40% or so). On the other hand, NAD–GDH activity is essentially close to the assay detection limit for the control strain, whereas, H1803 has a fairly high specific activity for this NAD–enzyme.

These results show that the recombinant strain H1803 overexpressing the NAD–glutamate dehydrogenase has significantly enhanced capabilities for ethanol (and xylitol) production, both in terms of higher specific productivities as well as higher product yields on the carbon substrate. The recombinant strain also produces significantly less (undesired) cell mass and very substantially less (undesired) carbon dioxide, thereby not only increasing yields of desired products but also decreasing disposal and pollution loads.

**Example 9. Ethanol Production from Glucose**

Glucose fermentation to ethanol was conducted in 1.8 liter Chemap CMF Fermentor (Switzerland) by genetically engineered strains of *Saccharomyces cerevisiae*, designated as H1793 and H1791, both of which are derived from *S. cerevisiae* CEN.PK2 (VW–1B) (Boles *et al.* [1996]). Strain H1791 is transformed with a plasmid that expresses the NAD–glutamate dehydrogenase (NAD–GDH) from *S. cerevisiae* (YEplac181+*GDH2*), whereas strain H1793 contains only the cloning vector (YEplac181) and serves as a

control strain. Plasmid segregation can be minimized by omitting leucine (LEU) from the growth media.

The inoculum was prepared by transferring a single colony into a 250 ml Erlenmeyer flask that contained 50 ml of modified synthetic complete medium without leucine (SC–LEU) + 20 g/l of glucose. The cells were grown overnight on a rotary shaker at 150 rpm and 30 °C ($OD_{600}$: 10–15). Cells from the above cultivation were harvested by a 10 minute centrifugation at 4,500 rpm and 4°C, washed with 0.1 M $PO_4^{2-}$ buffer (pH=5.5) and resuspened in the same buffer each to a final volume of 25 ml and subsequently transferred to the fermentor. The fermentation medium contained (per liter): yeast–nitrogen–base (without amino acids and without ammonia) 3.4g, uracil 0.044g; tryptophan 0.164g, histidine 0.116g, $KNO_3$ 5.055g, glucose 40g. The fermentor temperature was maintained at 30 °C, the pH was controlled at 5.5 by addition of 2 M NaOH, and the agitation was constant at 300 rpm. The cultivation was carried out under anaerobic conditions by flashing the headspace of the fermentor with nitrogen at a constant flowrate of 0.2 vvm. The offgas was connected via a multi–port valve to a Balzers quadrupole mass spectrometer (Sweden) for online analysis. Liquid samples were withdrawn from the fermentor at time intervals to measure growth, substrate consumption, and the formation of extracellular products. Biomass, glucose, ethanol, glycerol and acetate were measured as in the previous example.

Table 1 summarizes the primary fermentation data for these two fermentations. The NAD–GDH overexpressing strain (H1791) accumulates on the average about 12% less biomass (0.52 vs. 0.46 g/l). during the 21 hour time period. Specific glucose consumption rates are comparable for the two strains (within 5%). However, the *GDH2* strain has both a higher volumetric (11%) and a higher specific (25%) ethanol production rate. Yet another extraordinary divergence between the two recombinant strains is depicted in Figure 8. This carbon dioxide data comes from the mass spectrometer measurements of the effluent gas. As shown in Figure 8, overexpression of NAD–GDH significantly attenuates carbon dioxide evolution. Integrated values for $CO_2$ production from 0 to 21 hours are 93.7 and 70.6 mmol/l for the control and *GDH2* strains respectively, i.e. the *GDH2* strain wastes about 25% less of the carbon source to carbon dioxide. The specific $CO_2$ production rate for the *GDH2* strain is also attenuated by

about 15%. In addition, the yield of ethanol on glucose (mol/mol) is estimated to be approximately 19% higher for the *GDH2* strain vs. the control strain.

These results show that the recombinant strain H1791 overexpressing the NAD-glutamate dehydrogenase (NAD-GDH) has significantly enhanced capabilities for ethanol production from glucose, both in terms of higher specific productivities as well as higher product yields on the carbon substrate. The recombinant strain also produces significantly less (undesired) cell mass and less (undesired) carbon dioxide, thereby not only increasing yields of desired products but also decreasing disposal and pollution loads.

**Table 1.** Glucose fermentations with *Saccharomyces cerevisiae* recombinant strain overexpressing NAD-GDH (H1791) and control strain (H1793): comparison of growth, glucose consumption, ethanol production and carbon dioxide evolution rates. The last two rows show calculated average fluxes expressed in either volumetric ($J_V$, mmol/l h) or specific ($J_S$, mmol/g-cell h) terms. Glucose and ethanol concentrations represent average values from four measurements: two with HPLC and two with enzymatic assays.

| | *Biomass (g/l)* | | *Glucose (g/l)* | | *Ethanol (g/l* | | *$CO_2$ (mole %)* | |
|---|---|---|---|---|---|---|---|---|
| *Time (h)* | 1793 | 1791 | 1793 | 1791 | 1793 | 1791 | 1793 | 1791 |
| 0 | 0.315 | 0.300 | 39.27 | 37.57 | 0.01 | 0.01 | 0.000 | 0.000 |
| 6 | 0.740 | 0.670 | 37.68 | 36.48 | 0.63 | 0.72 | 1.333 | 0.619 |
| 17 | 0.790 | 0.760 | 33.00 | 32.49 | 2.39 | 2.37 | 1.552 | 0.823 |
| 19 | 0.820 | 0.690 | 32.22 | 31.04 | 2.65 | 2.78 | 1.735 | 0.701 |
| 21 | 0.800 | 0.670 | 30.98 | 29.85 | 2.98 | 3.30 | 1.818 | 0.711 |
| $J_V$ | - | - | -2.19 | -2.04 | 3.08 | 3.41 | 4.46 | 3.36 |
| $J_S$ | - | - | -4.21 | -4.43 | 5.92 | 7.40 | 8.58 | 7.31 |

**References**

Amore *et al.* (1989). Appl. Microbiol. Biotechnol. 30: 351–357.

Avendano A, Deluna A, Olivera H, Valenzuela L and Gonzales A (1997). J. Bacteriol. 179: 5594–5597.

Beier DR and Young ET (1982). Characterization of a regulatory region upstream of the *ADR2* locus of *S. cerevisiae*. Nature 300: 724–728.

Bergmeyer (1974). Methods of Enzymatic Analysis; Academic Press.

Björkqvist *et al.* (1997). Appl. Environ. Microb. 63: 128–132.

Boeke JD, LaCroute F and Fink GR (1984). A positive selection for mutants lacking orotidine–5'–phosphate decarboxylase activity in yeast: 5–fluoro–orotic acid resistance. Mol. Gen. Genet. 197: 345–

Boles E, Lehnert W and Zimmermann FK (1993). The role of the NAD–dependent glutamate dehydrogenase in restoring growth on glucose of a *Saccharomyces cerevisiae* phosphoglucose isomerase mutant. Eur. J. Biochem. 217: 469–477.

Boles E, Göhlmann HWH and Zimmermann FK (1996). Cloning of a second gene encoding 6–phospho–fructo–2–kinase in yeast, and characterization of mutant strains without fructose–2,6–bisphosphate. Mol. Microbiol. 20: 65–76.

Bruinenberg *et al.* (1983). Eur. J. Microbiol. Biotechnol. 18: 287–292.

Bruinenberg (1986). Antonie von Leeuwenhoek 52: 411–429.

Chen *et al.* (1994). FEBS Lett. 356: 81–85.

Chen *et al.* (1997). Eur. J. Biochem. 250: 578–582.

Coschigano PW, Miller SM and Magasanik B (1991). Mol. Cell. Biol. 11: 4455–4465

Courchesne WE and Magasanik B (1988). J. Bacteriol. 170: 708–713.

Dang V–D, Bohn C, Bolotin–Fukuhara M and Daignan–Fornier B (1996). J. Bacteriol. 178: 1842–1849.

Follettie, M. T., Archer, J., Peoples, O. P. & Sinskey, A. J. (1991). Metabolic Engineering of Corynebacteria. *In* Societe Microbiologique Francaise. Edited by H. Heslot, J. Davies, J. Florent, L. Bobichon, G. Durand & L. Penasse. Paris.

Follettie, M. T. & Sinskey, A. J. (1986). Recombinant DNA Technology for *Corynebacterium glutamicum*. Food Technology 40: 88.

Fuck E, Stärk G und Radler F (1973). Äpfelsäurestoffwechsel bei *Saccharomyces*. II Anreicherung und Eigenschaften eines Malatenzyms. Arch. Microbiol. 89: 223–231.

Gietz RD and Sugino A (1988). New yeast–*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six–base pair restriction sites. Gene 74: 527–534.

Gietz D, St. Jean A, Woods RA and Schiestl (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20: 1425.

Hill J, Donald KAIG and Griffiths DE (1991). DMSO–enhanced whole cell yeast transformation. Nucleic Acids Res. 19: 5791.

Hirose (1986). Biochemical Effects of Oxygen Supply and Carbon Dioxide Removal *in* Biotechnology of Amino Acid Production, ed. K. Aida, I. Chibata, K. Nakayama, K. Takinami & H. Yamada. Tokyo: Kodansha – Elsevier. 24: 67–80.

Ho *et al.* (1983). Biotechnol. Bioeng. Symp. 13: 245–250.

Jetten, M. S. M., Follettie, M. T. & Sinskey, A. J. (1994). Metabolic Engineering of Corynebacterium glutamicum. In Recombinant DNA Technology II, pp. 12–29. Edited by R. Bajpai & A. Prokop. New York: Annals New York Academy of Science.

Jetten, M. S. M. & Sinskey, A. J. (1995). Recent Advances in the Physiology and Genetics of Amino Acid–Producing Bacteria. Critical Reviews in Biotechnology 15: 73.

Kaplan (1960). page 150 *in* The Enzymes, 2nd edition, ed. Boyer, Lardy, Myrbäck; Academic Press.

Kötter and Ciriacy (1993). Appl. Microbial. Biotechnol. 38: 776–783

Lagunas *et al.* (1976). Mol. Cell. Biochem. 13: 73–78

McAlister–Henn L, Steffan JS, Minard KI and Anderson SL (1995). J. Biol. Chem. 270: 21220–21225.

Mellor J, Dobson MJ, Roberts, NA, Tuite MF, Emtage JS, White S, Lowe PA, Patel T, Kingsman AJ and Kingsman SM (1983). Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. Gene 24: 1–14.

Metzger and Hollenberg (1995). Eur. J. Biochem. 228: 50–54.

Metzler (1977). page 466 *in* Biochemistry: the chemical reactions of the living cell; Academic Press

Miller SM and Magasanik B (1991). Mol. Cell. Biol. 11: 6229–6247.

Minard KI and McAlister-Henn L (1992). J. Biol. Chem. 267: 17458-17464.

Neidhardt, F. C., Ingraham, J. L. & Schaechter, M. (1990). Physiology of the Bacterial Cell: a Molecular Approach. Sunderland, MA: Sinauer Associates, Inc.

Oura (1972). page 30, Doctoral dissertation, University of Helsinki

Oura (1977). Process. Biochem. April issue

Rose M, Grisafi P and Botstein D (1984). Structure and function of the yeast *URA3* gene: expression in *Escherichia coli*. Gene 29: 113-124.

Ruohonen L, Aalto M and Keränen S (1995). Modifications to the *ADH1* promoter of *Saccharomyces cerevisiae* for efficient production of heterologous proteins. J. Biotechnol. 39: 193-203.

Sarthy *et al.* (1987). Appl. Environ. Microbiol. 53: 1996-2000.

ter Schure EG, Silljé HHW, Verkleij AJ, Boonstra J and Verrips CT (1995). J. Bacteriol. 177: 6672-6675.

Sherman, F., Fink, G. & Hicks, J. B. (1983). Methods in Yeast Genetics. A Laboratory Manual. Cold Springs Harbor, N.Y.: Cold Springs Harbor Laboratory.

Tantirungkij *et al.* (1994). Appl. Microbiol. Biotechnol. 41: 8-12.

Toikkanen J, Söderlund H. and Keränen S. (1998). Genetic interactions between the early and late secretory pathway in *Saccharomyces cerevisiae*. Manuscript in preparation.

Verduyn, C., van Kleff, R., Schreuder, H., van Dijken, J. P. & Scheffers, W. A. (1985). Properties of the NAD(P)H-dependent Xylose Reductase from the Xylose Fermenting Yeast Pichia stipitis. Biochemical Journal 226: 669-677.

Viljoen M, Subden RE, Krizus A and van Vuuren HJJ (1994). Molecular analysis of the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*. Yeast 10: 613-624.

Walfridsson *et al.* (1995). Appl. Environ. Microbiol. 61: 4184-4190.

Walfridsson *et al.* (1996). Appl. Environ. Microbiol. 62: 4648-4651.

**Claims**

1. A microorganism transformed with at least one recombinant DNA molecule encoding or otherwise causing the expression of at least one enzyme that facilitates an oxidation–reduction reaction between the NAD/NADH and NADP/NADPH coenzyme couples, said transformed microorganism thereby producing useful products more efficiently than a corresponding non–transformed microorganism.

2. The microorganism of claim 1, said microorganism producing more product per unit of raw material than does a corresponding non–transformed microorganism.

3. The microorganism of claim 1, said microorganism producing a product faster than does a corresponding non–transformed microorganism.

4. The microorganism of claim 1, said microorganism producing less $CO_2$ per unit of a product produced than does a corresponding non–transformed microorganism.

5. The microorganism of claim 1, said microorganism having a reduced oxygen requirement per unit of a product produced than has a corresponding non–transformed microorganism.

6. The microorganism of any one of claims 1 to 5, wherein the product is ethanol.

7. The microorganism of claim 6, wherein the ethanol is derived from a pentose.

8. The microorganism of any one of claims 1 to 5, wherein the product is one or more amino acids.

9. The microorganism of any one of claims 1 to 8, wherein at least one of the recombinant DNA molecules encodes or otherwise causes the expression of an enzyme which is a dehydrogenase.

10. The microorganism of claim 9, wherein the dehydrogenase is selected from the group consisting of glutamate dehydrogenases, malic dehydrogenases, malic enzymes, isocitric dehydrogenases and aldehyde dehydrogenases.

11. The microorganism of any one of claims 1 to 10, which microorganism is a yeast.

12. The microorganism of claim 11, which microorganism is a strain of *Saccharomyces* spp., *Schizosaccharomyces* spp. or *Pichia* spp.

13. The microorganism of any one of claims 1 to 10, which microorganism is a bacterium.

14. The microorganism of any one of claims 1 to 5 and 8 to 10, which microorganism is a strain of Corynebacteria or Brevibacteria.

15. *Saccharomyces cerevisiae* strains selected from the group consisting of H1791 (VTT C–98298), H1795 (VTT C–98300), H1803 (VTT C–98302), H1840 and H1842.

16. A method of producing ethanol from raw materials, comprising fermenting said materials with a microorganism of any one of claims 1 to 7, 9 to 13 and 15.

17. A method of claim 16, wherein the raw materials comprise pentoses, pentose polymers or mixtures thereof.

18. A method of producing one or more amino acids, comprising treating raw materials with a microorganism of any one of claims 1 to 5 and 8 to 15.

**(57) Abstract**

The present invention relates to genetic engineering of production microorganisms used in biotechnology to improve their properties so that they produce useful products more efficiently. In particular the invention relates to increasing the yields of products such as ethanol or amino acids from carbon and nitrogen sources such as biomass comprising hexoses, pentoses or their polymers.

Fig. 1

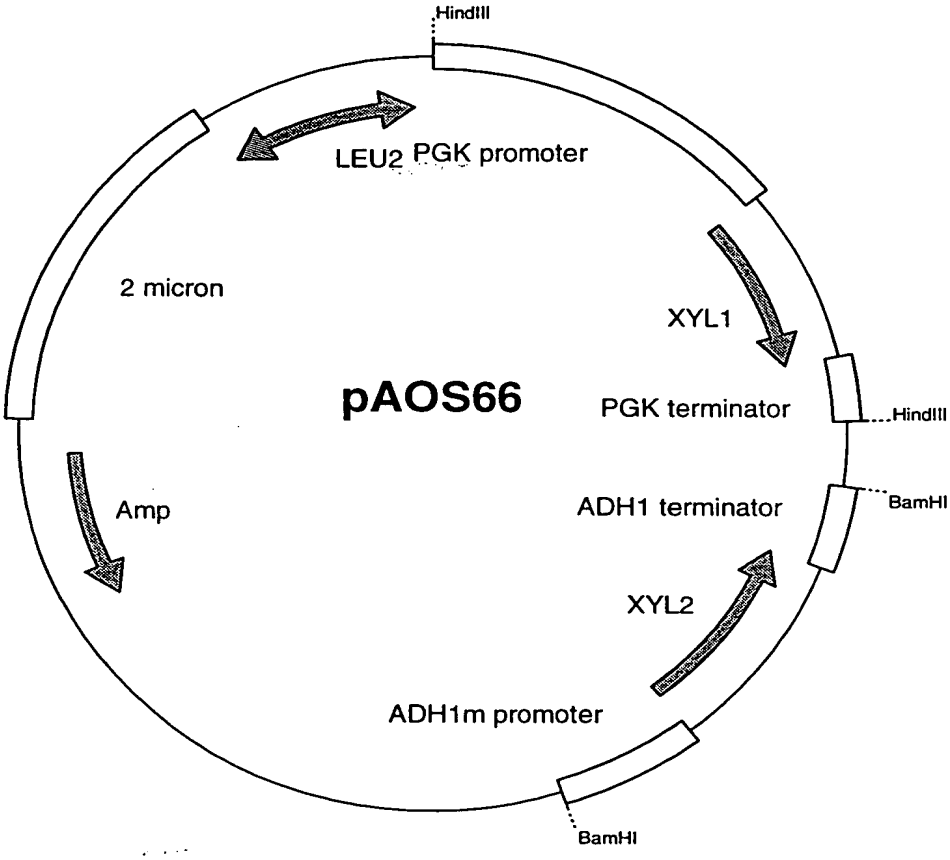
HindIII
LEU2
PGK promoter
2 micron
XYL1
pAOS66
PGK terminator
HindIII
Amp
ADH1 terminator
BamHI
XYL2
ADH1m promoter
BamHI

Fig. 2

Fig. 3

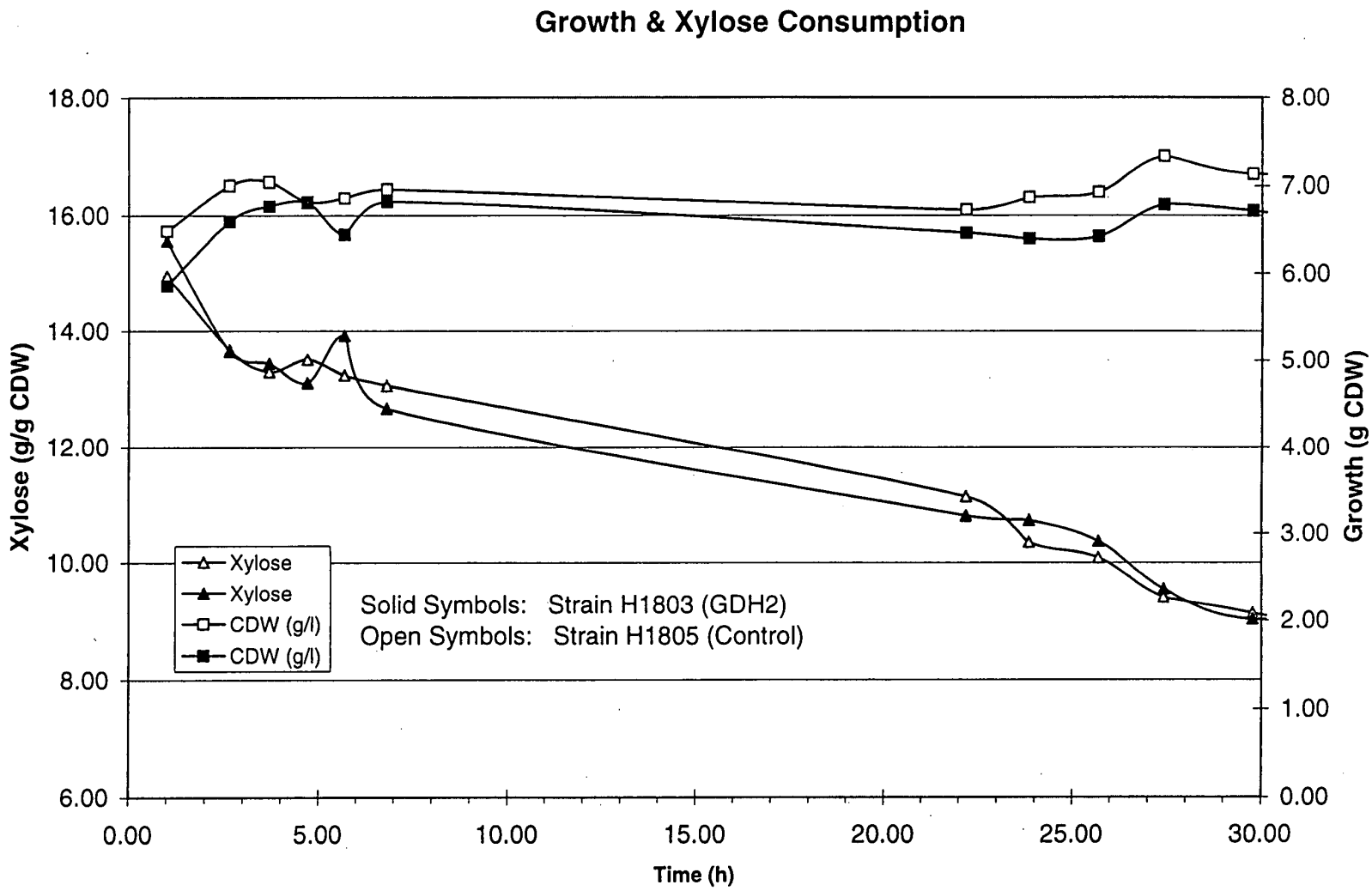
Growth & Xylose Consumption
18.00
16.00
14.00
12.00
10.00
8.00
6.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Xylose (g/g CDW)
Growth (g CDW)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Time (h)
Xylose
Xylose
CDW (g/l)
CDW (g/l)
Solid Symbols: Strain H1803 (GDH2)
Open Symbols: Strain H1805 (Control)

Fig. 4

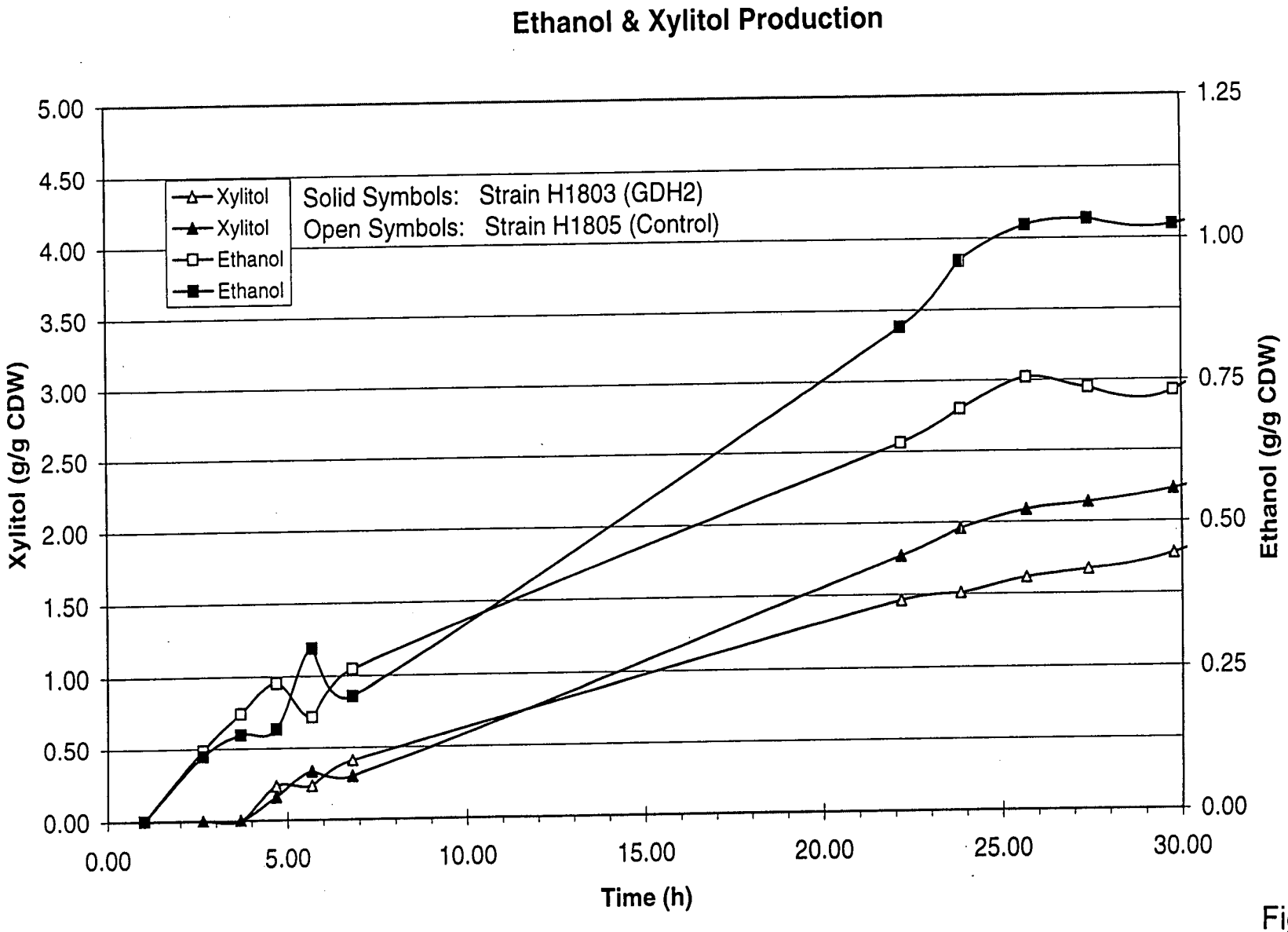
Ethanol & Xylitol Production
Xylitol
Xylitol
Ethanol
Ethanol
Solid Symbols: Strain H1803 (GDH2)
Open Symbols: Strain H1805 (Control)
Xylitol (g/g CDW)
Ethanol (g/g CDW)
Time (h)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1.25
1.00
0.75
0.50
0.25
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00

Fig. 5

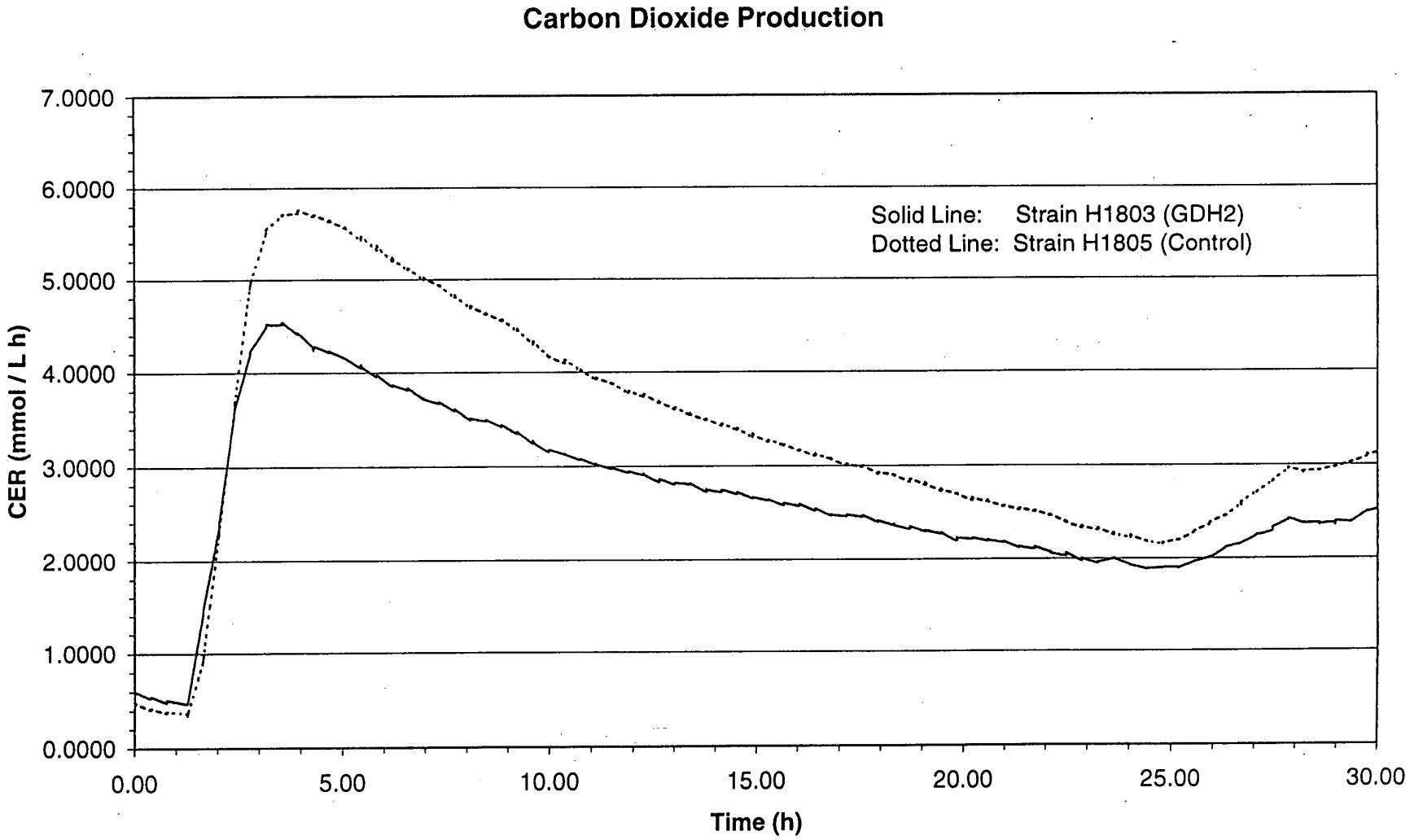
Carbon Dioxide Production
7.0000
6.0000
5.0000
4.0000
3.0000
2.0000
1.0000
0.0000
CER (mmol / L h)
Solid Line: Strain H1803 (GDH2)
Dotted Line: Strain H1805 (Control)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Time (h)

Fig. 6

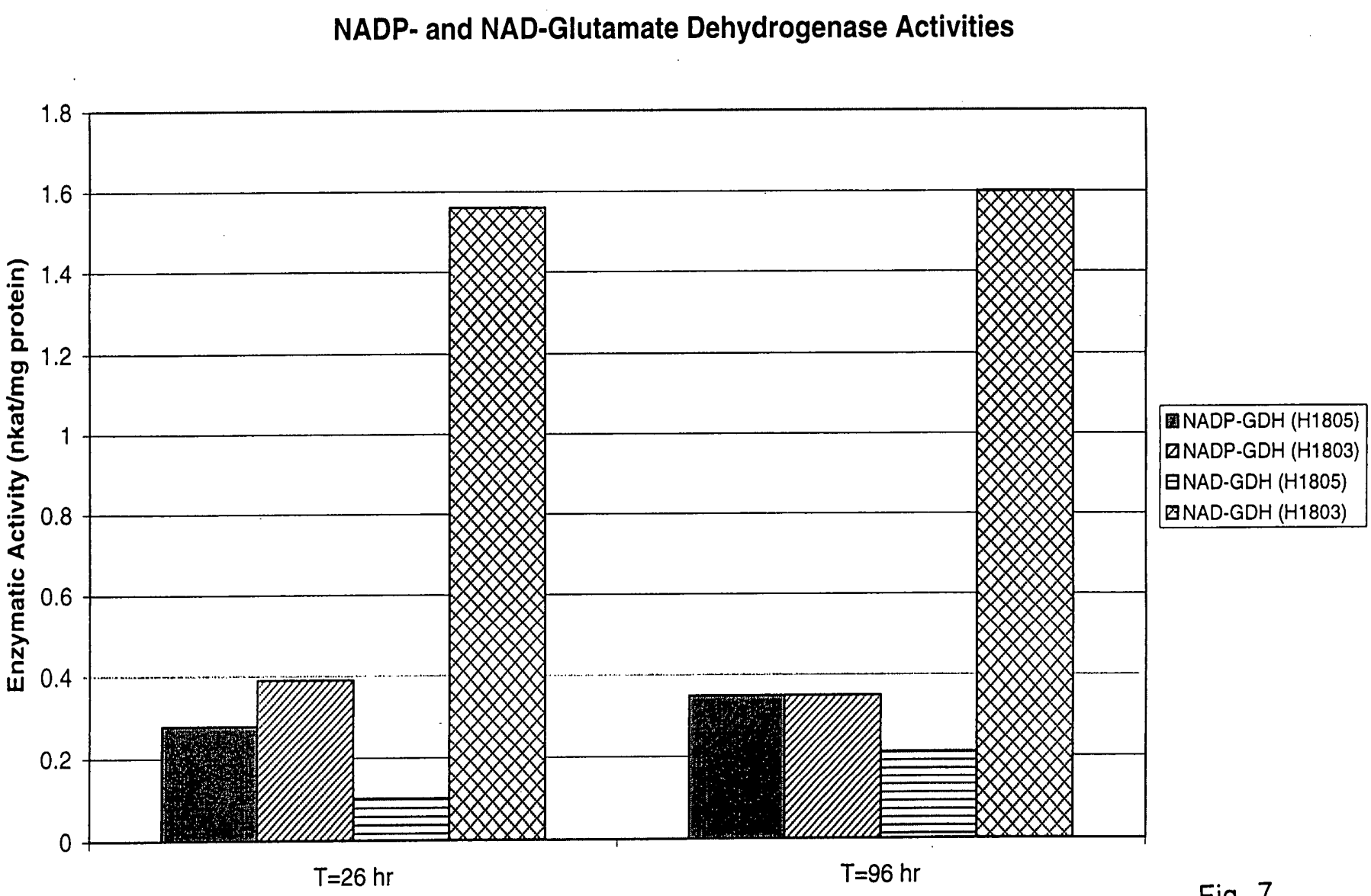
NADP- and NAD-Glutamate Dehydrogenase Activities
Enzymatic Activity (nkat/mg protein)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
T=26 hr
T=96 hr
NADP-GDH (H1805)
NADP-GDH (H1803)
NAD-GDH (H1805)
NAD-GDH (H1803)

Fig. 7

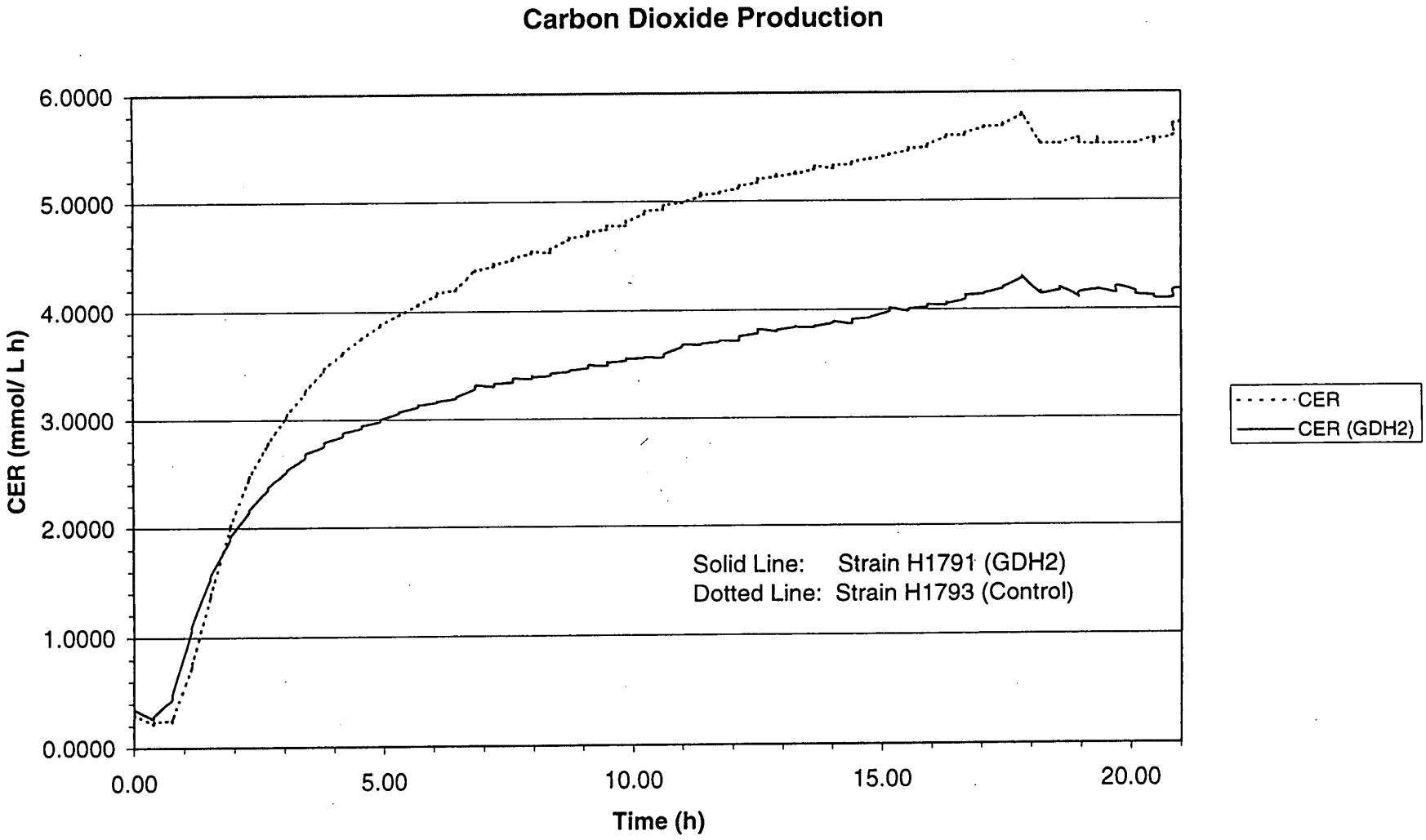
Carbon Dioxide Production
6.0000
5.0000
4.0000
3.0000
2.0000
1.0000
0.0000
CER (mmol/ L h)
0.00
5.00
10.00
15.00
20.00
Time (h)
Solid Line: Strain H1791 (GDH2)
Dotted Line: Strain H1793 (Control)
CER
CER (GDH2)

Fig. 8